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Gene therapy in diabetes

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Abbreviations: AIA, adjuvant induced arthritis; APC, antigen presenting cell; AtT20 cells, mouse pituitary corticotroph cells; Bcl, B-cell CLL/lymphoma; BGL, blood glucose level; bp, base pairs; CCL, CC chemokine ligand; CD, cluster of differentiation; CMV, cytomegalovirus; CP, core peptide; CTB, cholera toxin B; CTLA, cytotoxic T-lymphocyte antigen; DC, dendritic cell; DKA, diabetic ketoacidocis; EAE, experimental allergic encephalomyelitis; EF1α, elongation factor-1α; eGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; Ex, exendin; FADD, fas-associated death domain; GAD, glutamic acid decarboxylase; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; h, hour; hHGF, hepatocyte growth factor; HLA, human leukocyte antigen; HSP, heat shock protein; IA-2, protein tyrosine phosphatase-like molecule; ICA512, protein tyrosine phosphatase-like molecule; ICAM, inter-cellular adhesion molecule; IDDM, insulin-dependent diabetes mellitus; IFN, interferon; Ig, immunoglobulin; IGF-I, insulin-like growth factor I; IL, interleukin; INS, insulin; IPF1, insulin promoter factor 1; IPGTT, intraperitoneal glucose tolerance test; LTR, long terminal repeat; mg, milligram; MHC, major histocompatibility complex; min, minutes; MIN, pancreatic β-cell line; μ, micro; μIU, micro international unit; μg, microgram; μL, microliter; mL, milliliter; ng, nanogram; NIT-1, pancreatic β-cell line; NK, natural killer; NOD, non-obese diabetic; ORF, open reading frame; PBS, phosphate buffered saline; PC^h, prohormone convertase; PC^P, proprotein convertase; PD-1-PD-L1, programmed death-1-programmed death ligand-1; PDX-1, pancreatic duodenal homeobox 1; PEI, polyethylenimine; rAAV, recombinant adeno-associated vector; rAD.A20, adenoviruses expressing A20; rAD.β-gal, adenoviruses expressing β-galactosidase; RIP-Tag, rat insulin promoter-SV40 T-antigen; RNA, ribonucleic acid; SCID, severe combined immunodeficiency; SIN, self inactivating vector; STC-1, murine enteroendocrine K cell-derived cell line; STZ, streptozotocin; SV40, simian virus 40; T1D, type 1 diabetes; TCR, T-cell receptor; TGF, transforming growth factor; TNF, tumor necrosis factor; TRADD, tumor necrosis factor receptor type 1-associated death domain; βtc-3, insulinoma cell line

Type 1 diabetes (T1D) is a chronic autoimmune disease, whereby auto-reactive cytotoxic T cells target and destroy insulin-secreting β-cells in pancreatic islets leading to insulin deficiency and subsequent hyperglycemia. These individuals require multiple daily insulin injections every day of their life without which they will develop life-threatening diabetic ketoacidosis (DKA) and die. Gene therapy by viral vector and non-viral transduction may be useful techniques to treat T1D as it can be applied from many different angles; such as the suppression of autoreactive T cells to prevent islet destruction (prophylactic) or the replacement of the insulin gene (post-disease). The need for a better method for providing euglycemia arose from insufficient numbers of cadaver islets for transplantation and the immunosuppression required post-transplant. Ectopic expression of insulin or islet modification have been examined, but not perfected. This review examines the various gene transfer methods, gene therapy techniques used to date and promising novel techniques for the maintenance of euglycemia in the treatment of T1D.

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Introduction

Diabetes mellitus is a clinical condition caused by insulin deficiency or a resistance to insulin, resulting in elevated blood glucose levels (BGLs; hyperglycemia). The consequences of long-term hyperglycemia can lead to end stage micro- and macrovascular damage leading to organ failure such as neuropathy, nephropathy, retinopathy, peripheral vascular disease, morbidity and mortality; reviewed by Sowers and Epstein, Klein and Vinik et al.[1-3] Diabetes mellitus is a devastating disease that can present at various ages, in different forms and can display a myriad of clinical presentations and features. The incidence of the disease is increasing worldwide and the most common diabetes category in the United States, Canada, Europe and Australia is type 2 diabetes (T2D), accounting for more than 80% of diabetes cases.[4] A further 5–10% of cases are reported as type 1 diabetes (T1D) and the remaining cases are due to other causes including gestational diabetes, endocrinopathies and post-viral infections.[4]

Although hyperglycemia is an indicator for both T1D and T2D, the clinical features and pathophysiology between the two disorders are vastly different.[5] Onset of T1D is generally within the first two decades of life, hence the former name juvenile diabetes. Approximately one quarter of the cases have been noted to develop later in life.[6] With the discovery of autoantibodies, 7.5–10% of T2D cases are T1D that present during adult years.[7-9]

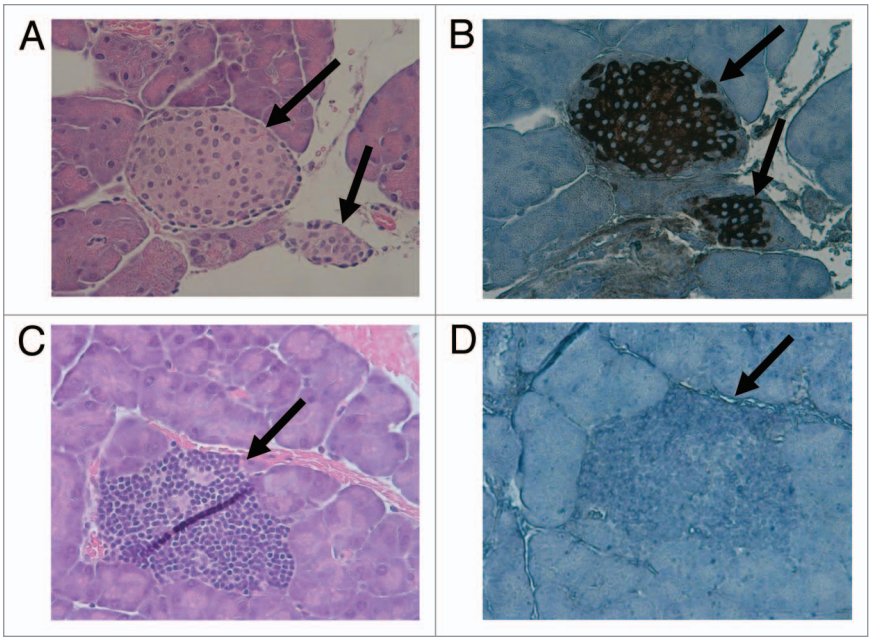

Figure 1. Pancreatic tissue showing islets stained with H&E and insulin from untreated NOD mice. (A) H&E stain of non-diabetic pancreas from control group. (B) Insulin stain (brown) of non-diabetic pancreas from control group. Islets are shown by arrows. (C) H&E stain of diabetic pancreas from control group. Lymphocytes infiltrating the islet are shown by the arrow. (D) Insulin stain of diabetic pancreas from control group. The arrow points to the absence of insulin producing cells.

Epidemiology. In autoimmune diseases females are generally more affected than males. However, in the majority of populations studied there is no change in incidence of T1D between genders. An Australian study of children under 15 years of age reported a higher incidence of T1D in females than males.[10] Furthermore, in the Jamaican population, there was a 2:3 male to female ratio for T1D.[11] However, these findings contrast with other populations in which males were more prone to develop T1D than females. A 3:2 male to female ratio was seen in European populations between 15 to 40 years of age[12] and a similar finding was reported in a Boston study of children under 6 years old.[13] In the United States, T1D accounts for approximately two thirds of the newly diagnosed cases of diabetes in individuals under 19 years old.[14-17] The peak age for T1D onset is between 4 and 6 years, with another peak occurring between 10 and 14 years of age.[18-20]

Incidence of diabetes (both type 1 and type 2) worldwide is 246 million.[21] The incidence rate for T1D in America is 23.6 per 100,000 persons.[22] Increased incidence of T1D is noted worldwide with an annual increase in Europe, Middle East and Australia of 2, 5 and 3% respectively.[12,23-27] The diagnosis of T1D has been noted in more than 13,000 children and adolescents under the age of 19 per year.[28] The prevalence in America is 2.0 per 1,000 persons.[22]

The risk of developing T1D increases as the distance from the equator increases.[29] This observation is supported by data showing people developing T1D when relocating from low incidence areas to high incidence areas. Countries with the highest reported incidence of T1D are Finland and Sardinia[30] and the lowest incidence is in Asia.[31] Not all similar geographical areas display similar incidences of T1D. This indicates that environmental factors may also affect the incidence of the disease. Viral infections, immunizations, diet, early exposure to cow's milk, maternal age, history of pre-eclampsia and neonatal jaundice all increase the risk of T1D. By contrast, low birth weight decreases the disease risk.[32] Different climates are thought to influence the incidence of T1D. However studies are contradictory[33,34] and no conclusion can yet be made as to whether or not climate alters T1D incidence rates.

Studies from the United States indicate that certain ethnic groups have higher incidence rates when compared to others. The highest incidence occurs in Caucasian youth followed by African American and Hispanic youth.[35] The lowest incidence is in Asian/Pacific Islanders and American Indians.[35]

Pathophysiology. T1D manifests when there is destruction of at least 70–90% of insulin-producing β-cells (insulitis) by an inflammatory infiltrate of cluster of differentiation (CD)8^+ and $CD4^+$ T cells, B cells, macrophages; with a predominance of $CD8^+$ T cells (**Fig. 1C and D**).[36-38] Previous studies suggest that complement-mediated lysis and Fas-Fas-ligand binding triggered apoptosis of inflamed islets.[36,39] This can be compared to **Figure 1A and B**, which depicts normal Islets of Langerhans without cellular infiltrate. The hypothesis for the aetiopathogenesis of T1D is outlined in **Figure 2**.[40] In association with the cellular infiltrate there are auto-antibodies produced to several pancreatic islet auto-antigens in approximately 85% of individuals with T1D.[41] The main auto-antibody detected is against glutamic acid decarboxylase (GAD65). Other auto-antibodies include protein tyrosine phosphatase-like molecule (IA-2 or ICA512) and insulin[42] that serve as biomarkers for the disease.[38] For example, the presence of anti-GAD65 antibody in a healthy individual or diabetic is an indication that the individual may require insulin in the future.

Therapy. In adults and adolescents, randomized trials have conclusively established that poor glycemic control is associated with long term vascular sequelae. The secondary complications include nephropathy, retinopathy, neuropathy and cardiovascular disease and have been previously reviewed in ref. 43. As such there is a need for earlier intervention.

Genetic Engineering

Genetic engineering can occur by one of two possible methods—germ line and somatic manipulation. Genes from germ line genetic manipulation are transferred to the individual's offspring whereas somatic genetic manipulation will only affect the individual to

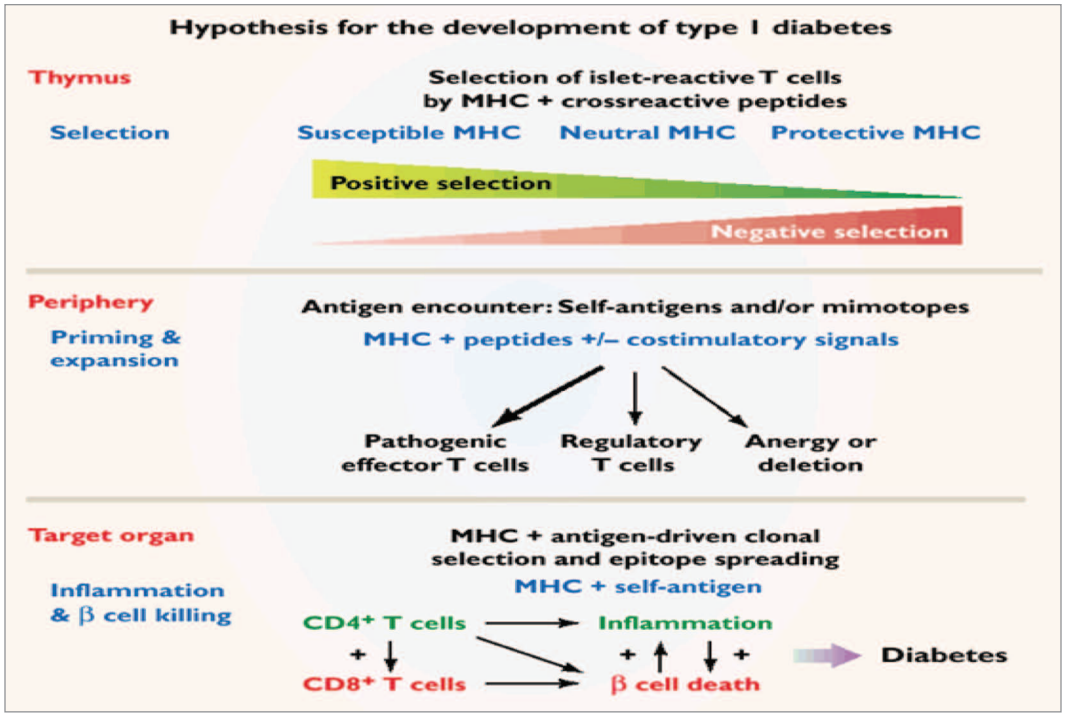

Figure 2. Antigen-presentation events in the development of T1D. A number of different MHC haplotypes influence susceptibility to type 1 diabetes in humans, and confer differing degrees of susceptibility or resistance. These MHC molecules could affect thymic selection by promoting positive and/or negative selection of islet-reactive T cells. The initial encounter of naïve islet reactive T cells with antigen may occur outside the target organ and could be driven by self-antigens and/or cross-reactive microbial antigens. At the effector stage, both $CD4^+$ and $CD8^+$ T cells contribute to insulitis and β cell loss. $CD8^+$ T cells lyse β cells in a perforin-dependent manner, whereas $CD4^+$ T cells can kill β cells by secretion of cytokines such as TNFα. Presentation of islet antigens is enhanced by upregulation of MHC class I and II expression and antigen release, which leads to activation of T cells with additional specificities (epitope spreading). Diabetes results when the majority of β cells have been lost. In humans, β cells are typically lost over the course of several years, offering opportunities for therapeutic intervention. Reproduced with permission from Wucherpfennig and Eisenbarth 2001.[40]

which the transgene is introduced. Gene transfer can be divided into in vivo or in vitro transfer. For successful in vivo delivery, the vehicle for the transgene must be appropriately directed to the target cells and the gene product must be protected from immune attack. Manipulating cells genetically in vitro is less invasive than in vivo techniques however target cells are required to be easily removed and transplanted back into the host.

Gene therapy. In T1D, islets are the target for autoreactive T cell destruction. The absence of islets leads to insulin deficiencies and resultant hyperglycemia. Gene therapy is a useful technique to treat T1D as it can be applied from many different angles. The insulin gene can be replaced in a host or the autoreactive T cells suppressed. These and many other various techniques are discussed below.

Gene transfer methods. A number of various gene transfer methods have been used. These include non-viral methods such as calcium phosphate co-precipitation, lipofection, direct microinjection, electroporation and biolistics, as well as gene transfer via viral vectors.

Non-viral methods. Calcium phosphate co-precipitation is a simple and non-expensive method for genetically modifying pancreatic cells. When calcium chloride with the DNA of interest is added to buffered saline/phosphate solution, a precipitate forms. Cells can endocytose or phagocytose the DNA-containing precipitate. This method has been tested in a variety of cell types and can produce either transiently transfected cells or cells that are able to stably express the transgene. Liposomes have also been used as high efficiency transfection agents of cells both in vivo and in vitro; unlike calcium phosphate co-precipitation, which is conducted in vitro. The advantage of in vivo lipofection is that the liposomes may be injected into the bloodstream and is less invasive than other treatments, such as transplantation. Liposomes containing DNA have minimal positive charges which improve their interaction with target cells and the consequent transfection efficiency.[44] Directly injecting DNA into cells is an effective method for transfecting cells. However, as each cell needs to be targeted individually, this is a labor intensive technique and is not suited for the targeting of large cell numbers. Electroporation creates permeable membranes for gene transfer by applying high voltages to cells; and in many cases, causes cell death. To allow efficient gene transfer to surviving β-cells the islets need to be dissociated from the tightly clustered sacs of cells into single cell suspensions. Without the maintenance of their morphology, the dissociated islets may be non-functional. Although it is possible for gene transfer into the cell, electroporation cannot efficiently integrate DNA into the host genome.[45] In comparison to both lipofection and calcium phosphate co-precipitation, biolistic transfection produces higher transfection efficiencies. Biolistics is the use of a "gene gun" to transfect cells with a transgene.[45] The "gene gun" rapidly discharges DNA—microprojectiles into cells.

Viral vectors. The choice of an appropriate vector requires careful consideration. In order to be successful vectors need to be simple to manufacture in large numbers, have the ability to be targeted to a specific site, be able to transduce both dividing and non-dividing cells, result in high transduction efficiency, not elicit a strong immune response and allow for long term expression of the transgene.[46] For transgene delivery into islets, the vector is required to pass through the islet membrane and transduce the sac of cells within. Studies by Leibowtz et al. have previously shown that successful transduction of the cells within islets only occur at the periphery of the islet (approximately 10% of cells) and cells in the core of the islet are not transduced.[47] The main disadvantage of retroviral transduction is that they are only able to transduce cells that are currently dividing—non-dividing islets cannot be transduced by retroviral vectors.[48] There may also be random integration of the transgene into the host genome, resulting in insertional mutagenesis.[49]

Adenoviral vectors have the advantage over retroviral vectors in that they are able to transduce both dividing and non-dividing cells[48] and can be prepared in high titres.[50] Adenoviruses can infect insulin-secreting cells[47] and have been shown to be able to transduce rodent islets.[51-53] Barbu et al. have shown that by confocal sectioning of intact islets transduced with GFP that expression on the cells was in fact only on the periphery of the islets and as such transduction efficiencies are approximately only 30%.[54] The weaknesses of this type of gene transfer are that the vector antigens elicit potent immune responses[55] and the inserted DNA is episomal, resulting in short term transgene expression.[56]

Lentiviral vectors have similar characteristics to both retroviral vectors and adenoviral vectors. The retroviral characteristics are the ability to integrate the transgene into host chromosomal DNA and to alter the surface envelope proteins. Lentiviral vectors are able to transduce primary and post-mitotic cells—such as neurons, liver, muscle cells, primary endothelial cells and islets;[47,57] and to transduce dividing and non-diving cells without the potent immune responses that adenoviral vectors elicit.[49]

Recently, lentiviral vectors have been produced without genes that are unnecessary for transduction and this decreased the possibility of active recombinant virus production.[49] Self inactivating vectors (SINs) are unable to produce a full length vector[49] because there is a 400 base pair (bp) deletion within the U3 region of the 3' long terminal repeat (LTR).[55] It has also been noted that the insertion of a 178 bp fragment amplified from HIV-1 strain NL4-3 between the *rev* response element and the internal CMV promoter resulted in enhanced transduction efficiency.[55]

Gene therapy treatments. Maintenance of euglycemia can be achieved by a variety of genetic manipulations; including tolerance induction, interference with antigen presentation, interference with co-stimulation of T cells, use of immunoregulatory cells, the induction of apoptosis, ectopic gene expression, transplantation and immunosuppression.

Treatments for normalising blood glucose levels following islet destruction. Ectopic gene expression. The targeted cell type in T1D is the β-cell and some gene therapy treatments attempt to re-create the functions of the β-cell in cell types not targeted by the immune system. The expression of genes in cell types that are not their usual area of expression is termed ectopic gene expression. Ectopic expression of genes is a widely used technique and circumvents the requirement of immunosuppressive measures because the target for genetic engineering originates from the recipient of the graft and may be an unlimited source of autologous cells. Important features of β-cells which are essential in the regulation of blood sugar levels include continuous monitoring of glucose levels, regulated transcription and translation of pro-insulin, regulated pro-insulin processing to mature insulin, regulated storage of mature insulin and regulated secretion of mature insulin to a stimulus, such as glucose.[58,59] Good alternatives to β-cells for manipulation into insulin producing cells include hepatocytes, fibroblasts, muscle, keratinocytes, neuroendocrine cells and many other endocrine cells.[45]

Ectopic insulin expression has been trialed in cells such as mouse pituitary corticotroph (AtT20 cells) in 1983,[60] and fibroblasts in 1987.[61] AtT20 cells are similar to β-cells in that they are able to secrete proinsulin and also express the proconvertases, proprotein convertase (PC^P)2 and PC^P3, to convert proinsulin into mature insulin.[50] However, since AtT20 cells are a different cell type from β-cells the expression of insulin in AtT20 cells confirmed that non-β-cells could be modified to secrete insulin. For this finding to be useful, however, target cells for genetic engineering must have a regulated secretory pathway. Fibroblasts were engineered to express pro-insulin under a metallothionein promoter, but insulin production was constitutive and animals transplanted with the modified fibroblasts died from hypoglycaemia stemming from lack of regulated insulin release.

(1) Epithelial cells. Epidermal keratinocytes are self-renewing cells and are an unlimited source of autologous cells, unlike islets. With the use of a lentivirus consisting of CMV promoter Tian et al. genetically engineered these cells to produce a fusion protein, when stimulated by rampamycin, consisting of the proinsulin that has been modified to allow cleavage by furin and a self-dimerisation mutant of FK506-binding protein to generate bioactive insulin.[62] During the process of insulin production by islets, proteases (prohormone convertases; PC^h1/PC^h3 and PC^h2) cleave the disulfide bonds linking C-peptide to the A-chain and B-chain, releasing C-peptide and mature insulin. However, in cells such as keratinocytes or hepatocytes, the proteases required for producing mature insulin are absent. By altering the human insulin gene to produce a recognition site for a propeptide endoprotease which is abundant in the Golgi apparatus (furin), liver cells were allowed to process proinsulin into mature insulin. The keratinocytes were monitored in vitro for insulin secretion when stimulated with rampamycin. Within 30 min after rampamycin addition, insulin was detected in the supernatant and within 2–3 h of the removal of rampamycin, insulin was no longer detected in the supernatant. Transduced keratinocytes were also evaluated for their insulin expression in vivo, by transplantation into athymic streptozotocin (STZ)-induced diabetic mice. The mice were stimulated with rampamycin and 1 h following there was insulin detected in the plasma until 2–4 h post-injection of rampamycin. Control mice without the rampamycin stimulation or without the transduced cells did not express insulin in the plasma.

Prior to Tian et al. Lei et al. also modified keratinocytes to ectopically express proinsulin with furin recognition sequences between C-peptide and the A- and B-chains, but with a recombinant retrovirus, pBMNIG, instead of a lentivirus.[63] Transduced cells were able to produce proinsulin and this was quantified by enzyme-linked immunosorbent assay (ELISA). The measurement of proinsulin conversion to mature insulin could not be quantified from measuring the supernatant as with the proinsulin measurement. For the keratinocytes to proliferate the presence of mature insulin is required and the insulin added to the media cannot be distinguished from the insulin exported from transduced cells. The measurement of C-peptide, the product released from the conversion of proinsulin to mature insulin, by ELISA was performed as an alternative. The levels of both proinsulin and C-peptide were high, suggesting that keratinocytes are good candidates for ectopic gene expression of insulin.

K cells are a different type of enteroendocrine epithelial cell that are located in the intestinal lining. They have similar characteristics to β-cells and are a good candidate for modification. Zhang et al. genetically modified a murine enteroendocrine K cell-derived cell line, STC-1, with a plasmid encoding insulin under the control of a GIP promoter (pcDNA3-GIP-hIns).[64] STC-1 cells were transduced to produce insulin and initially their insulin-producing capability was assessed by measuring insulin secretion when the STC-1 cells were placed in a high glucose (from 1 mM to 10 mM) media. After an increase in insulin was noted (10.8 ± 0.8 to 23.6 ± 2.3 μIU/mL) in response to increased glucose levels, the clone STC-1-14 was transplanted into STZ-induced diabetic nude mice. After injection with STZ, BGL of the mice increased over 16.7 mM and gradually began to lose weight. BGLs in all mice transplanted with engineered STC-1 cells achieved euglycemia and regained lost body weight 26 days following post-transplant and remained so until the end of the study at 49 days post-transplant.

(2) Hepatocytes. Hepatocytes are similar to β-cells in the sense that they can facilitate the removal of glucose from the bloodstream via phosphorylation by glucokinase in response to high glucose levels.[59] They are good candidates for genetic manipulation because they are the essential regulators of carbohydrate metabolism via insulin[58] and are already equipped with the machinery to respond to fluctuating glucose levels and can be modified to produce insulin as glucose levels increase. Many different research groups have targeted hepatocytes for modification with either an insulin gene or a gene that will promote insulin expression. Olson et al. examined the use of an adenovirus vector with an insulin transgene under the control of a glucose and insulin responsive, liver-specific promoter.[65] Diabetes in BBDR/Wor rats was induced by inter-peritoneal injections of poly-I:C with the virus administered via the jugular vein. When comparing rats that had been administered the virus compared to the control, BGLs of treated rats returned to normal levels, metabolic switching returned to normal and there were reduced intra-abdominal fat deposits. Whereas the control rats remained severely hyperglycemic.

Ren et al. and others have also transduced hepatocytes to produce insulin but the difference is that the insulin gene has been modified with furin recognition sequences. A lentivirus with an HIV/murine stem cell virus hybrid long terminal repeat promoter was used by Ren et al. to transduce hepatocytes to express insulin with furin-recognition sequences.[66] Transduction efficiencies were tested both in vitro and in vivo and were found to be greater than 87 1.2% and up to 60 3.2% respectively. Transduced cells were administered by intervallic infusion in full flow occlusion to the livers of STZ-induced diabetic rats. Following transduction, an immediate reduction of BGLs was seen in the diabetic rats, and within 5 days, BGLs of diabetic rats were comparable to non-diabetic healthy rats. BGLs of control rats that were transduced with an empty lentiviral vector remained high until the end of study at day 60. This study was the first to demonstrate long term normalization of BGLs of STZ-induced diabetes with the use of a lentiviral delivery system of furin-cleavable insulin.

Hsu et al. used a recombinant adeno-associated vector (rAAV) and the rat insulin I promoter to drive the expression of furin-mutated human insulin in Huh7 human hepatoma cells.[67] Transduced Huh7 cells were assessed for insulin production in response to glucose in vitro by culturing the Huh7 cells in media with varying concentrations of glucose. The amount of fluorescent protein in the supernatant, which is indicative of the amount of insulin produced by the cells, was analyzed by flow cytometry. The effect of polyethylenimine (PEI) on rAAV-mediated gene transfer was assessed and PEIs were found to enhance the expression of insulin in vitro. STZ-induced diabetic C57BL/6J mice were infected with rAAV-PEI complexes by direct injection into the liver. The amount of insulin the mice produced in response to glucose was evaluated by an intraperitoneal glucose tolerance test (IPGTT). In mice treated with rAAV-PEI, euglycemia was achieved; contrasting with the untreated control group, where BGLs remained high. Although the rAAV-PEI treated diabetic mice achieved normal BGLs, there was a delay of approximately 30 min to achieve normal BGLs when glucose-stimulated, compared to healthy non-diabetic mice. This delay may be caused by delayed regulation of transcription of insulin in mice treated with rAAV-PEI compared with healthy mice. The time for insulin release by hepatocytes needs to be shortened so that this method can be able to function close to the speed of natural insulin production, where islets only have to release stored insulin.

The use of hepatocytes to secrete insulin is not ideal due to the time delay stemming from their inability to store insulin. Endocrine cells have the ability to regulate the secretion of various molecules and are able to store insulin in their secretory granules. However, these cells are still functionally different from the β-cell because they are not able to secrete molecules in response to stimuli, such as glucose.[59]

Instead of ectopically expressing insulin, Fodor et al. investigated the outcome of transplantation of hepatocytes that had been transduced with a lentivirus encoding an elongation factor-1α (EF1α) promoter and the human pancreatic duodenal homeobox 1 [PDX-1; also known as insulin promoter factor 1, homeodomain transcription factor (IPF1)] gene.[68] PDX-1 is essential for pancreatic development and drives the production of insulin.[69] Transduced hepatocytes were cultured with glucose and the insulin produced was measured. In response to stimulation with

25 mM glucose, insulin released was noted as 48±16 ng per mg of protein of cell lysate per 2 h. The cells were also transplanted into STZ-induced diabetic SCID mice. Following the transplant, the mice were challenged with an intra-peritoneal injection of glucose and the BGLs were measured. BGLs fell from 30.7±1.3 mM to 8.7±3.7 mM within 8 weeks and this was thought to be due to the transplanted hepatocytes, as removal of the graft resulted in recurrence of hyperglycemia.

(3) Stem cells. Stem cells have many characteristics that make them attractive for use as surrogate β-cells. They have the ability to multiply in culture, unlike islets which do not differentiate. Stem cells have also been suggested as being immune-privileged, so as to overcome the autoimmune response that destroy islets.[70] Xu et al. studied the transduction of murine mesenchymal stem cells by a recombinant retroviral plasmid that had been subcloned with an insulin gene under the control of the CMV promoter; and the effect of the transduced cells on STZ-induced diabetic mice.[70] The modified stem cells were transplanted into mice by intrahepatic injections. Mice treated with the stem cells, had lowered BGLs by the end of the study at 42 days. Treated mice BGLs were 6.5±0.89 mM compared with untreated mice, which had BGLs of 25.4±4.1 mM. At the end of the study, the mice were sacrificed and the livers were frozen sectioned for histological analysis. The distribution of the stem cells were viewed under a fluorescent microscope as green fluorescence and the presence of insulin was visualized as a brown stain after staining with an anti-human insulin polyclonal antibody. This data suggests that murine mesenchymal stem cells successfully expressed human insulin and was able to maintain normoglycemia for at least 42 days.

Systemic gene expression. Rather than transplant one specific cell type, Shternhall-Ron et al. systemically administered PDX-1 with the use of a recombinant adenovirus with a CMV promoter to redirect the functionality of hepatocytes to that of pancreatic cells.[71] The animal model that was used was cyclophosphamide-accelerated diabetes in NOD mice. This model of liver-to-pancreas redirection has been shown to be successful in chemically induced diabetes. The use of the cyclophosphamide-NOD model, a more natural representation of the course of the disease, was to assess whether liver-to-pancreas redirection can occur when the pancreas is under autoimmune attack. The adenovirus encoding PDX-1 was injected into the tail vein of cyclophosphamide-induced diabetic mice. Of the mice that became normoglycaemic, 43% of the PDX-1 treated mice exhibited a reversal of hyperglycemia; whereas untreated mice maintained high BGLs. Positive detection of the endocrine hormone (insulin, glucagon and somatostatin) gene expression was seen in more than 80% of PDX-1 treated mice, indicating the redirection of hepatocyte-to-islet like function. Insulin was detected at a 55-fold increase in PDX-1 treated mice when compared with non-treated mice. In treated mice, 17.75±7 ng insulin per liver was detected whereas only 0.325±0.175 ng insulin per liver was detected in non-treated mice. Hence, redirection of hepatocyte-to-islet like function is a possible treatment for T1D.

Nanoparticles. Even though many of the gene transfer techniques described here have used viral vectors, some laboratories make use of the safer non-viral gene delivery methods such as chitosan nanoparticles. Niu et al. wrapped an expression plasmid, pCMV.Ins (human insulin gene under the control of a CMV promoter), with chitosan nanoparticles and this was transfected to rats via the gastrointestinal tract (lavage and coloclysis).[72] Following treatment, the BGLs of rats decreased from 22.12 1.31 mM to 5.63±0.48 mM (lavage group) and 5.07±0.37 mM (coloclysis group). Hence, treatment with the human insulin gene expression vector wrapped in chitosan nanoparticles is a possible non-viral method for treatment of hyperglycemia.

Non-viral technique. Soltani et al. transferred glucagon-like peptide-1 (GLP-1)/IgG-Fc fusion protein to muscle cells of STZ-induced diabetic mice with the use of electroporation, also a non-viral technique for gene transfer.[73] GLP-1/IgG-Fc, as well as an analogue of GLP-1—exendin-4 (Ex4)/IgG-Fc, was subcloned into a vector (VRnew; derivative of the VR1255 vector) containing a CMV immediate-early enhancer-promoter. Mice received the vector through intramuscular DNA injections followed by electrical current locally applied to the skin. Compared with the IgG-Fc control group, both GLP-1/IgG-Fc and Ex4/IgG-Fc pretreated groups of STZ-induced diabetic mice exhibited an approximate 50% decrease in the incidence of T1D. Treatment with either GLP-1/IgG-Fc or Ex4/IgG-Fc was shown to improve β-cell numbers following depletion by STZ injections and increase insulin levels. STZ injections decreased β-cell numbers by approximately 75% and increased by approximately 280% after GLP-1/IgG-Fc or Ex4/IgG-Fc treatment, which was still approximately 30–40% less than that of mice that received neither STZ, GLP-1/IgG-Fc nor Ex4/IgG-Fc. This data suggests that GLP-1 and its analogue Ex4 may be used as a treatment for T1D and other diseases where there is β-cell injury.

Engineered islets. Transplantation of any organ or tissue, other than autografts, requires some protection from the immune system. Protection of grafts can be achieved by various methods, some of which will be described in the following section: Preventative treatments. Methods include grafting into immunologically privileged sites (brain, thymus or testes); isolation from the immune system (encapsulation); inducing immune tolerance to grafts; or prevent immune attack by altering the grafts in one of two methods: (a) graft modification to prevent recognition of the graft as foreign; (b) graft modification to allow the graft to induce inactivation or apoptosis of the immune cells.[45]

Allo-transplantation of human islets is one method of returning BGL to normal levels. Donors for these transplants are cadavers and the availability of islets is dependent on the limited organ numbers. Large quantities of functioning islets are required for amelioration of T1D after transplantation; however the process of islet isolation and purification destroys many of the essential microvasculature for islet survival. In an attempt to repair the routes of nutrient and oxygen delivery, Panakanti and Mahato transduced islets with an adenovirus under the control of a CMV promoter to express hepatocyte growth factor (hHGF)—which increases the growth and proliferation of β-cells and IL-1 receptor antagonist (hIL-1Ra)—which prevents islet apoptosis.[74] They produced a virus Adv-hHGF-hIL-1Ra, an adenovirus that

is able to transduce cells to express HGF and IL-1Ra under a CMV promoter. Transduced human islets, expressing both HGF and IL-1Ra, affected the Bcl-2 gene (anti-apoptotic) positively and Bax gene (pro-apoptotic) negatively; and showed that the presence of HGF and IL-1Ra promoted the reduction apoptotic pathways. Monitoring of caspase 3 levels showed that there was not an increase of caspase 3 levels when stimulated with a cytokine cocktail containing IL-1β, tumor necrosis factor (TNF)α and interferon (IFN)γ. Furthermore, the diminished quantity of caspase 3 lead to decreased apoptosis of islets and the normalization of BGLs for more than 28 days post-transplant when the transduced islets were transplanted under the kidney capsule of STZ-induced diabetic NOD.SCID mice compared to the untransduced islet control.

Xeno-transplantation of non-human islets, such as porcine islets, can overcome the limited availability of human islets although the disadvantage of xenografts is that a greater immune response needs to be overcome when compared to the immune response to human cells.[45]

Human β-cell lines are also unlimited sources of islets, being able to differentiate and will elicit a reduced immune response compared to xenografts, however the ability to differentiate appears to retard insulin biosynthesis and regulation[58] and they will also be vulnerable to autoimmune attack. As β-cell lines have been modified with oncogenes, there is also a possibility of tumor formation.[45] Two cells lines that have been used previously are the MIN cell line and the NIT-1 cell line. The MIN cell line was derived from transgenic mice that expressed the SV40 T-antigen gene from an 1,867 bp human insulin promoter fragment.[45] MIN6 cells were capable of releasing insulin in response to glucose but could not transcribe insulin in response to glucose from the insulin gene in a controlled manner. The NIT-1 cell line is an insulinoma derived from NOD mice that expressed rat insulin promoter-SV40 T-antigen (RIP-Tag) transgene.[45] These cells are not targeted by T cells because they do not express major histocompatibility complex (MHC) on their cell surface in the absence of IFNγ, and only expresses MHC class I when there is IFNγ present.[45] At low passage number NIT-1 cells regulates release of insulin in response to glucose, however as passage number increases, insulin release is constitutive[45] and results in death of immune-compromised recipients.[75]

Prevention of islet destruction. The use of preventative treatments require advanced techniques capable of identifying the genetically susceptible individuals at risk of developing T1D. An example of previous screenings for genetically susceptible individuals was shown in results from genome-wide scans that assessed the relationship between T1D and genetics. These scans showed that the human leukocyte antigen (HLA) locus on chromosome 6q21.3 induces the highest susceptibility to T1D.[76] More specifically, the reports by Golden et al. have suggested that the HLA haplotypes DR3-DQB1*0201 and DR4-DQB1*0302 increase susceptibility to T1D.[77] DR3 and DQB1*0302 are thought to be the primary alleles linked with increased susceptibility and that DQB1*0201 and DR4 genes play secondary roles.[77] In cases where the disease was unable to be identified early, the following techniques—induction of immune tolerance, interference with antigen presentation, interference with activation or co-stimulation of T cells, immunoregulatory cytokines and apoptosis—may also be used as protective measures for transplanted islets.

Induction of immune tolerance. To prevent the immune system from recognizing and attacking our organs and cells, all T cells are screened in the thymus. Any T cell that reacts to presented self-antigens are killed. Since T1D occurs when the screening mechanism fails, one treatment for T1D is to induce tolerance to islets by introducing islet antigens to the thymus. Introduction of islet antigens to the thymus can be achieved by genetic manipulation of vectors to encode islet antigens, followed by injection into the thymus.[56]

Another method for inducing tolerance to an antigen has been suggested to be by association with stem cells. Tian et al. used a retrovirus (MMP-IAβ-d-GFP) encoding MHC class II I-A β-chain molecules—which have been suggested to be protective against T1D, to transduce autologous stem cells.[78] Control stem cells were transduced with MMP-GFP—without the protective MHC class II molecule. Following transplantation of the transduced stem cells, the programmed death-1-programmed death ligand-1 (PD-1-PD-L1) pathway was blocked in an attempt to accelerate the onset of T1D in mice. It has previously been shown that blockage of the PD-1-PD-L1 pathway precipitates diabetes in an accelerated fashion.[78] Mice were treated with anti-PD-L1 monoclonal antibody or with the control rat IgG antibody. In mice treated with both MMP-GFP transduced stem cells and anti-PD-L1, 75% of mice developed diabetes within 85 days. Conversely, BGL of mice treated with both MMP-IAβ-d-GFP and anti-PD-L1 remained normal for more than 180 days (end of study). MMP-IAβ-d-GFP (or MMP-GFP) was also transplanted into mice diabetic NOD mice who then received an islet transplant. BGLs of 5/6 mice that received MMP-IAβ-d-GFP returned to normal levels for more than 90 days (end of study), whereas mice that received MMP-GFP reached euglycemia within 24 h of transplant but became diabetic again within 6 days. The conclusion from this data is that treatment with MMP-IAβ-d-GFP has developed resistance to diabetes induced by blockage of the PD-1-PD-L1 pathway and also induced self tolerance to islet autoantigens.

Interference with antigen presentation. Antigen presenting cells (APCs), the dendritic cells (DCs), B-cells and macrophages, are responsible for activating T cells by presentation of the correct antigen. Prevention of interactions between the APCs and T cells inhibits T-cell activation and, in a T1D setting, halt the destruction of insulin-producing islets. The various points of intervention include the prevention of APC activation, the prevention of antigen processing by APCs, the reduction of adhesion molecule upregulation, the reduction of interactions between islets and secreted molecules of APCs, and the induction of T-cell death via interactions with genetically induced expression of Fas-ligand by APCs.[56] Some methods for prevention of islet death include inhibition of IL-1β production by macrophages—interactions between IL-1β and islets promote destruction of the islets via Fas-Fas-ligand apoptosis; or by blocking the cell-to-cell interactions between DCs and adhesion molecules, such as inter-cellular adhesion molecule (ICAM).[79-84]

Immunoregulatory cytokines. Immunoregulatory cytokines are another avenue for genetic modification in diabetes. Some have immunosuppressive properties, for example, IL-4 or IL-10; and other cytokines, such as transforming growth factor (TGF)β, can inhibit NK cell function, thymocyte proliferation, antibody production and T-cell activity.[56]

Creusot et al. transduced DCs with a lentivirus with IL-4, under the control of a cytomegalovirus (CMV) promoter.[85] Another study in their laboratory has found that there was a gradual decrease of IL-4 in the pancreatic lymph nodes of older non-obese diabetic (NOD) mice compared with IL-4 levels in pancreatic lymph nodes in diabetes-resistant NOD.B10 mice.[86] Creusot et al. attempted to reintroduce IL-4 back into diabetes-susceptible NOD mice and to monitor their BGLs. The transduced DCs were injected into the tail vein of 12 week old non-diabetic NOD mice and these were compared to mice injected with phosphate buffered saline (PBS) into the tail vein that were also injected when they were 12 weeks old and non-diabetic. 80% of the untreated group developed diabetes by 30 weeks compared with the DC/IL-4 treated group, where only 30% of mice developed diabetes by 35 weeks of age with a 4–8 week delayed onset of disease. It was also shown that MHC expression played a major role in the protective effect of IL-4 in diabetes. The BGLs of normal NOD mice were compared to the BGLs of MHC-deficient ($C2ta^{-/-}\beta2m^{-/-}$) mice when both groups received the same DC/IL-4 treatment. In the MHC-deficient group more than 70% of mice developed diabetes by 29 weeks of age, whereas only 20% of mice that exhibited normal MHC expression developed diabetes. These data indicate that IL-4 expression is able to protect NOD mice from diabetes when administered prophylactically, but the co-expression of MHC by DCs is also required.

Another group, Cameron et al. showed that following treatment with IL-4 in diabetic NOD mice the levels of intrapancreatic CC chemokine ligand (CCL)4 expression and intraislet CCL5 expression increases and decreases respectively.[87] Meagher et al. then showed that the protective effect of IL-4 is ineffective without the presence of CCL4.[88] T cells from the spleen of IL-4 treated (hence CCL4 treated) NOD mice were transferred to NOD.SCID mice. The NOD.SCID mice that were transferred T-cells from IL-4 treated mice had reduced incidence (4/10 mice) and delayed onset (98 days) of T1D compared with NOD.SCID mice that were transferred T-cells from NOD mice that were treated with the control vehicle (78 days). Following IL-4 treatment CCL4 was then sequestered from nine mice with the use of an anti-CCL4 antibody. All nine mice developed T1D within 66 days. Removing CCL4 enables the T-cells from IL-4 treated mice to regain their pathogenic ability and also shortens the time of onset of T1D. CCL4 was then cloned into a plasmid (pHERO8100) and was given to mice intradermally weekly from 3 weeks of age until destructive insulitis (14 weeks). In the control group, insulitis progressively worsened from 14 weeks until 33 weeks. In comparison, mice treated with pHERO8100-CCL4 exhibited less severe insulitis and the incidence of T1D was reduced from 72% to 27% by 35 weeks of age. This data suggests that CCL4 potentially can be used as a treatment for T1D.

Islet autoantigens, such as glutamic acid decarboxylase (GAD) and insulin (INS), conjugated with cholera toxin B (CTB) subunit have been shown to suppress T1D.[89-91] Denes et al. cloned the conjugates (CTB::GAD and CTB::INS) and IL-10 into recombinant vaccinia viruses, injected into 4 week old NOD mice and the BGLs monitored.[92] In the control group of untreated mice, by 29 weeks of age, 70% of them had developed diabetes. Comparatively only 30% of CTB::GAD treated mice and 40% of IL-10 treated mice developed diabetes. CTB::INS seemed to be a better treatment than CTB::GAD or IL-10, with only 20% of mice developing diabetes by 31 weeks of age and it seemed to delay the onset of diabetes when comparing treatment in 11 week old NOD mice and untreated controls. CTB::INS delivered by recombinant vaccinia virus may be a good treatment for T1D.

Apoptosis. Genetic manipulation of islets to express anti-apoptotic molecules can protect islets from apoptosis induced by cytotoxic agents. This can be achieved by islets expressing the anti-apoptosis gene—Bcl-2; interference with TNF, Fas, Fas-associated death domain (FADD) or tumor necrosis factor receptor type 1-associated death domain (TRADD) (apoptosis cascade); the use of enzymes that inactive free radicals that mediate apoptosis; or the use of heat shock proteins—HSP27 prevents apoptosis induced by nerve growth factor withdrawal and HSP70 prevents apoptosis induced by TNF and overexpression of caspase 3.[56]

Apoptosis induced by Fas contributes to the islet destruction and prevention of graft survival. Grey et al. examined a zinc finger protein, A20, shown to be an antiapoptotic TNFα-induced gene in endothelial cells.[93] Adenoviruses expressing either A20 (rAD.A20) or β-galactosidase (rAd.β-gal) were transduced into rodent islets. Islets were stimulated for apoptosis by IL-1β and IFNγ and significant levels of apoptosis were seen in islets transduced with rAd.β-gal and non-infected islets. Islets that were transduced with rAd.A20 exhibited similar levels of apoptosis as islets that were not stimulated with IL-1β and IFNγ; demonstrating the protective effect that A20 has on islet apoptosis induced by the cytokines.

To prevent Fas-mediated apoptosis, Klein et al. have transduced an anti-Fas tRNA-ribozyme also into an insulinoma cell line (βTC-3) under the control of a ribonucleic acid (RNA) polymerase III promoter.[94] The expression of the anti-Fas ribozyme was hypothesized to inhibit the expression of Fas, and hence islet apoptosis. Two ribozymes (Rz596 and Rz216) were designed, that were efficient at cleaving mouse Fas mRNA by identifying the triplets which are preferentially recognized by hammerhead ribozymes: GUC, CUC and GUA. The ribozyme Rz 596 was chosen for future experiments due to its higher efficiency at cleaving Fas mRNA. The chimeric tRNA-Rz596 was fused and transfected into βTC-3 insulinoma cells, NIT-1 cells and into dissociated primary single cell suspensions. To induce Fas expression in cells, the βTC-3 cells were stimulated with a combination of IFNγ and IL-1. The amount of Fas expressed by cells was assessed by immunostaining and flow cytometry. Immunostaining showed that the cells produced Fas when transduced with the tRNA-Rz596 compared with cells that were mock infected. Flow cytometry quantification demonstrated that cells

transduced with tRNA-Rz596 expressed an average of 80% less Fas than cells mock transduced. This trend was seen in all three cell types, indicating that ribozymes suppressing Fas expression may be a treatment for diabetes.

Insulin-like growth factor I (IGF-I) is known to inhibit IL-1β-mediated Fas-mediated apoptosis. Giannoukakis et al. transferred IGF-I by CMV driven adenoviral transfer to islets.[95] The islets were pretreated with IL-1β, to initiate the production of Fas, at concentrations sufficient to inhibit the function of islets at high glucose levels such as 18 mM. Islets that were transduced with IGF-I secreted higher amounts of insulin compared to untransduced control islets. The ability of IGF-I to suppress Fas-mediated apoptosis was assessed by monitoring the levels of caspase-3—a marker of the initiation of apoptosis. To induce apoptosis, IL-1β, along with an agonistic Fas antibody, was added to islets transduced with either IGF-I or eGFP (control). The level of caspase-3 activity was reduced in islets transduced with IGF-I compared to islets transduced with eGFP; and hence adenovirally transferred IGF-I is a technique for preventing apoptosis of islets induced by cytotoxic agents.

Kojima et al. took advantage of the property of neurons to maintain transferred genes for their lifetime and transduced ependyma cells by injection of a rAAV into the cerebroventricular cavity.[96] The rAAV encoded for leptin, a hormone which normally regulates energy homeostasis, but also has been shown in the hypothalamus to decrease tonic episodic insulin secretion and to reduce BGLs. STZ-induced diabetic mice were injected intracerebroventricularly with the rAAV encoding either GFP or leptin. BGLs were monitored and Kojima et al. found that mice injected with rAAV-GFP were hyperglycaemic and died by week 6 after injection. Following injections with rAAV-lep, the BGLs lowered in mice lowered to close to the levels of untreated mice. The results from this study suggest that leptin injected into the cerebroventricular cavity can ameliorate T1D.

Interference with activation or co-stimulation of T cells. The first stimulus for T-cell activation is the recognition of the self MHC molecules and the foreign antigen. The second stimulus is co-stimulation of B7-CD28 and is required for complete activation of helper T cells.

Co-stimulation and complete activation of T cells require interactions between B7 and CD28 molecules. Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) may be engineered into cells and be expressed. CTLA-4 is an analogue of CD28 and can competitively inhibit B7-CD28 interactions due to the preferential binding of B7 molecules for CTLA-4.[56]

Core Peptide

Core peptide (CP; GLRILLLKV) is an immunosuppressive peptide the sequence of which was first published in 1990 following studies on T-cell antigen receptor (TCR) assembly.[97] CP consists of two basic positively charged and hydrophilic amino acids (arginine and lysine) separated by four neutral and hydrophobic amino acids. The functional significance of this peptide was not published until 1997 when it was shown that CP inhibited T-cell activation at the TCR-αβ/CD3 chain interface.[98]

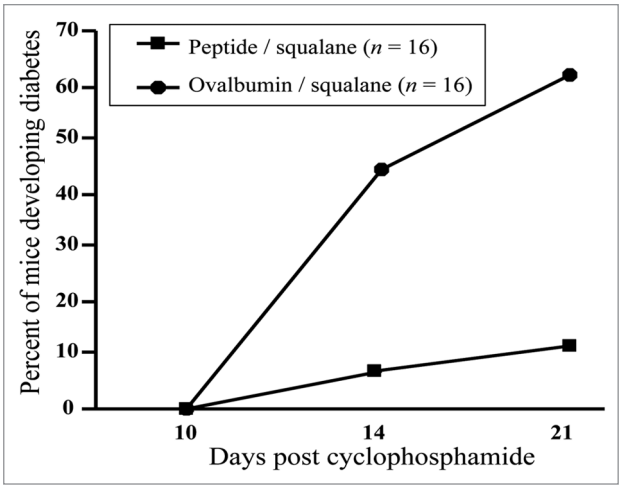

Figure 3. Subcutaneous injection of CP successfully suppresses T-cell activation in cyclophosphamide induced diabetes in NOD/Lt (F) mice. Reproduced with permission from Manolios et al. 1997.[98]

CP is T cell and natural killer (NK) cell specific with a minor effect on B-cells noted in higher concentrations.[99] In animal studies, CP given subcutaneously significantly reduced the induction of T cell mediated inflammation in adjuvant induced arthritis (AIA), experimental allergic encephalomyelitis (EAE) and delayed type contact hypersensitivity.[98] More recently it has been shown to be comparable in efficacy to cyclosporine in reducing the acute phase of inflammation in AIA.[100]

CP is believed to act at the transmembrane level, sterically inhibiting signal transduction. This view is supported by experimental results showing CP does not inhibit activation secondary to phorbol myristic acid (PMA)/ionomycin stimulation or CD3 cross-linking, suggesting that CP sterically inhibits the TCR-α chain or the -β chain CD3 interface.[101]

CP prophylactically prevents the induction of T1D in mice. Figure 3 demonstrates the protective effect of CP given to NOD (F) mice at the time of induction of diabetes with cyclophosphamide.[98] Mice were 10 weeks old at experimental day 0. CP was dissolved in squalane at a stock concentration of 3.33 mg/mL. Ovalbumin, used as a control was also suspended in squalane at the same concentration. A total of 250 μg (75 μL) was injected subcutaneously on the right flank on day -1, 0 and 1. Blood glucose measurements were taken on day 0, 10, 14 and 21. There were 16 mice in each of the CP and ovalbumin control groups. These experiments were performed "blind" at WEHI (Melbourne, Australia) by Prof L. Harrison. As shown, CP given at the same time as antigen reduced the induction of diabetes (prophylactically) from 60% down to 10% of the mice.

Dendritic cells engineered to secrete CP, induce antigen-specific immunosuppression in T-cell mediated disease. Using an adenoviral construct, dendritic cells (DCs) were transduced to secrete CP when injected into mice.[102] What was evident from these experiments was that CP can be engineered to cDNA and secreted by cells. The function of the peptide was not altered by genetic manipulation, and there was evidence of immunosuppression. In a CD8-driven allergy model, the injection of DCs

transduced with CP significantly reduced inflammation. In a $CD4^+$ T-cell dependent model of EAE, injection of CP secreting DCs abrogated symptoms and prolonged survival. These effects were antigen-specific since transduced DCs that did not express the respective antigen failed to convey protection. These experiments demonstrate that DCs engineered to secrete CP are able to suppress T-cell activation in an antigen specific and localized manner without affecting other immune cells or other cell types. These experiments provide strong evidence that CP is a very useful new agent for in vivo immune suppression.[102]

Conclusion

CP is a recently identified immunosuppressant peptide with the potential of being used as a single agent or as an adjunct to existing treatment protocols for the treatment of T1D. As a step towards therapy, CP could be engineered into cDNA and be lentivirally transduced into islets, which may then be transplanted into diabetic hosts. The continual expression of CP may prevent the cytotoxic destruction of islets by T cells and allow the maintenance of euglycemia—a treatment for T1D.

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